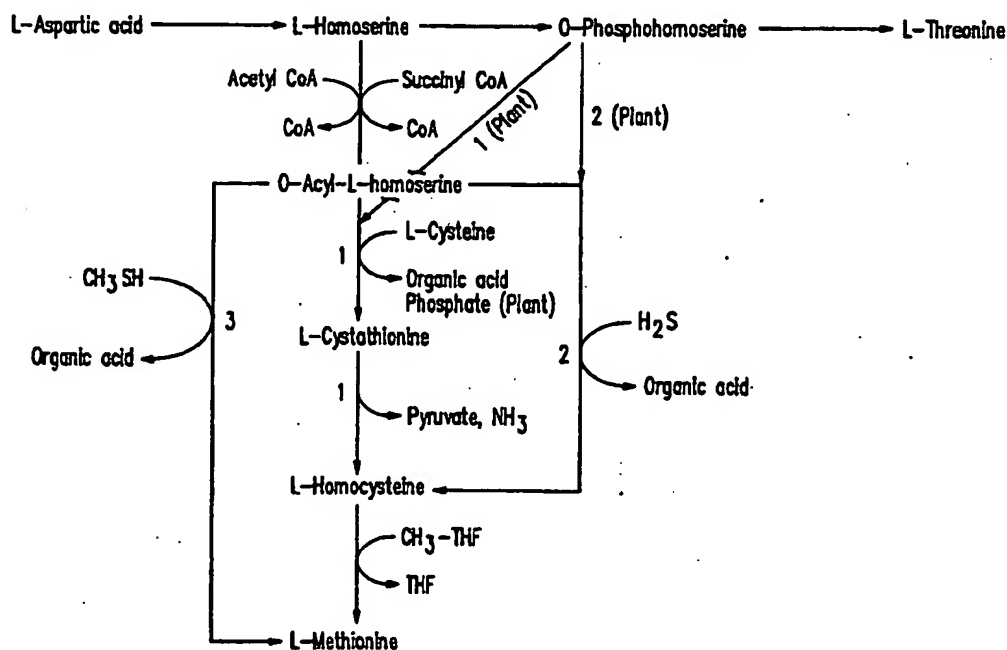




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/54, C12P 13/12 C12N 15/60, C12P 13/04		A1	(11) International Publication Number: WO 93/17112
			(43) International Publication Date: 2 September 1993 (02.09.93)
(21) International Application Number: PCT/US93/01351		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 16 February 1993 (16.02.93)		Published With international search report. With amended claims.	
(30) Priority data: 07/839,518 20 February 1992 (20.02.92) US			
(71) Applicant: GENENCOR INTERNATIONAL, INC. [US/US]; 180 Kimball Way, South San Francisco, CA 94080 (US).			
(72) Inventor: LIEVENSE, Jefferson, Clay ; 587 Pinegrove Avenue, Rochester, NY 14617 (US).			
(74) Agent: KRUPEN, Karen, I.; Genencor International, Inc., 180 Kimball Way, South San Francisco, CA 94080 (US).			

## (54) Title: BIOSYNTHESIS OF METHIONINE USING A REDUCED SOURCE OF SULFUR



## (57) Abstract

There are provided methods for the fermentation synthesis of methionine and homoserine using a reduced source of sulfur such as sulfide or methylmercaptan; and/or by modifying the methionine biosynthetic pathway in a producing microbe. Also provided are methods for the fermentation synthesis of methionine and homoserine using an oxidized sulfur source such as sulfate, sulfite or thiosulfate; and/or by modifying the methionine biosynthetic pathway in a producing microbe.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LJ	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

BIOSYNTHESIS OF METHIONINE  
USING A REDUCED SOURCE OF SULFUR

BACKGROUND OF THE INVENTION

Methionine is an essential amino acid in the diet of animals and is used widely as a food and feed supplement. It is conventionally produced by various multi-step chemical syntheses which generally employ acrolein, methyl mercaptan, and cyanide as starting materials. (H.H. Szmant, "Organic Building Blocks of the Chemical Industry," page 182, John Wiley & Sons, New York, 1989.) There are two resulting product forms: D,L-methionine and its hydroxy analog. Unlike all other amino acids, D-methionine is converted to the required L-form in vivo. As a result, chemical syntheses, which typically result in the D,L mixture, are feasible and cost-effective in this case.

However, fermentation production methods, which are common methods for making many low-cost amino acids, do not exist in the case of methionine. (K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada, "Biotechnology of Amino Acid Production," Progress in Industrial Microbiology 24, Elsevier, 1986.) This is surprising given that the biochemically related essential amino acids lysine and threonine are both produced cost-effectively by fermentation methods using inexpensive raw materials such as molasses, starch hydrolysates, corn steep liquor, and soy hydrolysates. (See for example: P.L. Rogers, R.G. Cail, D.F. Midgley, and C. Fryer, "The Prospects for L-Lysine Production in Australia," Food Technology in Australia 38, pp. 514-518, 1986; and S. Furukawa, A. Ozaki, and T. Nakanishi, "L-Threonine Production by L-Aspartate- and L-Homoserine-resistant mutant of Escherichia coli," Applied Microbiology and Biotechnology 29, pp. 550-553, 1988.)

Various microbes have been used to produce L-lysine and L-threonine. These have been developed through classical methods of mutagenesis and selection as well as genetic engineering. (K. Aida, supra.) Greatest success has been realized historically with the Corynebacteria and Brevibacteria, but it is also clear that other microbes such as Escherichia coli are viable.

There is a need for methods to reduce the metabolic cost and complexity of methionine biosynthesis, ideally making it similar to that for lysine or

threonine, such that an economical fermentation production of methionine is possible.

#### SUMMARY OF THE INVENTION

There are provided feasible fermentation methods for methionine synthesis comprising the use of reduced sulfur compounds instead of sulfate as the fermentation sulfur source and/or comprising re-designing and thereby simplifying the biochemical pathway. Also provided are fermentation methods for homocysteine synthesis comprising the use of reduced sulfur compounds instead of sulfate as the fermentation sulfur source and/or comprising redesigning and thereby simplifying the biochemical pathway. In a preferred embodiment of the present invention the reduced sulfur source is hydrogen sulfide, methyl mercaptan or salts thereof.

In another preferred embodiment of the present invention there are provided improved methods for such fermentation processes comprising re-designing or modifying and thereby simplifying the biochemical pathway.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is the common biosynthetic pathway to Lysine, Methionine and Threonine in Escherichia coli.

Figure 1b is the Threonine biosynthetic pathway in Escherichia coli.

Figure 1c is the Lysine biosynthetic pathway in Escherichia coli.

Figure 1d is the Methionine biosynthetic pathway in Escherichia coli.

Figure 2. Variations in the pathways for Methionine biosynthesis: (1) Transsulfurylation pathway; (2) Sulfhydrylation pathway; (3) Methylsulfhydrylation pathway.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for the fermentation synthesis of methionine and homocysteine. To understand why a cost-effective fermentation method for methionine synthesis does not exist, whereas such methods are available for lysine and threonine, it is instructive to consider in more detail the differences among the methionine, lysine, and

threonine biosynthetic pathways. All three amino acids are biochemically derived from the same intermediate metabolite, aspartic acid (Fig.1). In fact, threonine and methionine also share additional biochemical steps and the common intermediate homoserine. But the syntheses diverge substantially when their specific pathway branches are considered (Fig. 1). These are compared in Table I (J.L. Ingraham, O. Maaloe, and F.C. Neidhardt, "Growth of the Bacterial Cell," pp. 122-135, Sinauer Assoc., Inc., Sunderland, Mass., 1983.) The pathways present in *E. coli* are chosen as a basis of comparison, recognizing however that there is diversity in these pathways among microbes and plants and that this comparison should in no way be interpreted as limiting the present invention to pathways using *E. coli*. (K.M. Herrmann and R.L. Somerville, Chapters 9-13 in "Amino Acids: Biosynthesis and Genetic Regulation," Addison-Wesley Publishing Co., 1983; W.B. Jakoby and O.W. Griffith, Section III.D. in Methods in Enzymology 143, Academic Press, New York, 1987.)

Table I

Biochemical Building Blocks Needed to Synthesize  
Lysine, Threonine, and Methionine

<u>Amino Acid</u>	<u>Aspartate</u>	<u>Pyruvate</u>	<u>ATP</u>	<u>NADPH</u>	<u>1-C</u>	<u>S</u>
Lysine	1	1	2	3	0	0
Threonine	1	0	2	2	0	0
Methionine	1	0	7	8	1	1

It is evident from Table I that the biochemical energy requirements for methionine biosynthesis, in terms of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), are about three times higher than for lysine and threonine. This is due to the requirements of sulfate assimilation (J.L. Ingraham, *supra*.) A total of three moles of ATP and four moles of NADPH are required to biochemically reduce sulfate to sulfide. Two additional moles of ATP are required, one each to transport sulfate into the cell and to incorporate sulfide into cysteine. It is cysteine, finally, that serves as the sulfur donor in the biosynthesis of methionine (Fig. 1). In addition, methionine biosynthesis uniquely requires the incorporation of a methyl group (Fig. 1, Table I). This is derived as 5- methyl-tetrahydrofolate (CH<sub>3</sub>-THF) from the conversion of serine to glycine. Clearly considering the foregoing, the metabolic cost

and complexity of synthesizing methionine with sulfate as the sulfur source is much greater than that for lysine or threonine.

There is natural diversity among microbes and plants in the biosynthesis of methionine. This is represented schematically by Figure 2 and can be summarized as follows (K.M. Herrmann, *supra*; W.B. Jakoby, *supra*; F.C. Neidhardt, Chapter 27 in Escherichia coli and Salmonella typhimurium, American Society for Microbiology, Washington, D.C., 1987; M. Dixon and E.C. Webb, "Enzymes," 3rd edition, Academic Press, New York, 1979; S. Yamagata, Biochimie 71 (1989) 1125-1143):

- 1) In the methionine biosynthetic pathways of all microbes, homoserine is first activated either by succinyl-CoA (E. coli and S. typhimurium) or acetyl-CoA (fungi, yeast, and bacteria such as Brevibacterium and Bacillus). These reactions are catalyzed by homoserine succinyltransferase (EC 2.3.1.46) and homoserine acetyltransferase (EC 2.3.1.31), respectively.
- 2) In the methionine biosynthetic pathway of plants, homoserine is activated by ATP in a reaction catalyzed by homoserine kinase (EC 2.7.1.39). The homoserine kinase reaction also occurs in microbes, but the resulting O-phosphohomoserine is an intermediate in threonine, but not methionine, biosynthesis. Thus in plants O-phosphohomoserine is the branchpoint between the methionine and threonine pathways, whereas in microbes the branchpoint is homoserine.
- 3) In the microbial transsulfurylation route to methionine, acylhomoserine, in reactions catalyzed by O-succinylhomoserine (thiol)-lyase (EC 4.2.99.9) and cystathionine  $\beta$ -lyase (EC 4.4.1.8), accepts reduced sulfur from cysteine to give homocysteine. (O-Succinylhomoserine (thiol)-lyase is also known as cystathionine  $\gamma$ -synthase.)
- 4) In the microbial sulfhydrylation route, homocysteine is produced directly from acylhomoserine and sulfide by O-succinylhomoserine (thiol)-lyase or O-acetylhomoserine (thiol)-lyase (EC 4.2.99.10). O-acetylhomoserine (thiol)-lyase is also known as homocysteine synthase and methionine synthase.

- 5) In the microbial methylsulfhydrylation route, methionine is produced directly from acylhomoserine and methyl mercaptan by O-succinylhomoserine (thiol)-lyase or O-acetylhomoserine (thiol)-lyase.
- 6) The transsulfhydrylation and sulfhydrylation routes in plants are catalyzed by cystathionine  $\gamma$ -synthase. The plant enzyme cystathionine  $\gamma$ -synthase is distinct from EC 4.2.99.9 and is unique in using O-phosphohomoserine as a substrate.
- 7) Homoserine is a poor substrate of O-acetylhomoserine (thiol)-lyase, except in the case of the enzyme from Schizosaccharomyces pombe (S. Yamagata, supra).

The methionine biosynthetic enzymes above belong to the group of pyridoxal phosphate-containing enzymes. These are flexible catalysts known to carry out various elimination and replacement reactions. (C. Walsh, Chapter 24 in "Enzymatic Reaction Mechanisms," W.H. Freeman & Co., San Francisco (1979). Another of this group, tryptophan synthase converts serine and sulfide at a very high rate to cysteine (K. Ishiwata, T. Nakamura, M. Shimada, and N. Makiguchi, "Enzymatic Production of L-Cysteine with Tryptophan Synthase of Escherichia coli," J. Fermentation and Bioengineering 67: 169-172, 1989). This reaction is analogous with the reaction of homoserine and sulfide.

The various reactions relating to sulfur incorporation and methionine biosynthesis have yet to be considered in the design of a viable fermentation method. The use of sulfide or methyl mercaptan instead of sulfate reduces the metabolic cost of methionine synthesis to the levels of lysine and threonine. In the present invention two ATP and three NADPH are required since the active transport of sulfate, reduction of sulfate, and synthesis of cysteine are all eliminated.

Use of sulfide or methyl mercaptan also reduces the metabolic complexity of methionine biosynthesis since the biosynthesis of cysteine and, in the case of methyl mercaptan, CH<sub>3</sub>-THF are eliminated. Further simplification is possible and may be desirable by adapting the plant biosynthetic pathway to microbes by methods known to those skilled in the art. Since homoserine kinase is already present as an enzyme functioning in the microbial threonine pathway, this modification requires only introduction of plant

cystathionine  $\gamma$ -lyase activity. This could be accomplished by structurally modifying microbial O-acylhomoserine (thiol)-lyase or by expressing plant cystathionine  $\gamma$ -lyase in the producing microbe. Alternatively, structural modifications could be made in these enzymes or other candidate pyridoxal phosphate enzymes such as tryptophan synthase in order to effectively use homoserine directly as a substrate in sulfur incorporation. Or the O-acetylhomoserine (thiol)-lyase from *S. pombe* could be used without modification.

While reduced forms of sulfur would be preferred to minimize the requirement for biochemical energy, other more oxidized forms of sulfur are also beneficial. As described above, an improvement through metabolic simplification results whenever sulfide, rather than cysteine, is incorporated directly into homoserine or an activated derivative. Thus more oxidized forms such as sulfate, sulfite, and thiosulfate may be provided as sulfur sources and biochemically reduced to sulfide. Sulfite and thiosulfate also diminish the need for biochemical energy relative to sulfate since they are more reduced forms, although the energy requirement is greater than for sulfide or methyl mercaptan.

By reducing the complexity of the methionine biosynthetic pathway, the engagement of microbial metabolism in methionine over-production is less extensive. This reduces the number of genetic changes that must be introduced into the producing microbe by classical or genetic engineering methods in order to de-regulate methionine biosynthesis and limits the disruption of microbial metabolism, in general. As used herein, "de-regulate" means any effect on the self-regulation of the microbial metabolism for example, any effect on microbial self-regulation by feed-back inhibition or repression. Such de-regulation can be achieved through methods known to those skilled in the art such as for example, classical mutagenesis and selection or genetic engineering.

The net result is to transform the methionine biosynthetic pathway to one that compares favorably with those for lysine and threonine in terms of metabolic cost and complexity. In this way, a feasible fermentation method of methionine production can be realized.



## EXPERIMENTAL

The following disclosure is intended to serve as a representation of embodiments herein, and should not be construed as limiting the scope of this application.

### Example 1

#### Methionine Production via Acylhomoserine (Sulfhydrylation Route)

E. coli, C. glutamicum, and B. flavum are de-regulated for homoserine over-production by classical or genetic engineering methods. The sulfhydrylation route to methionine is introduced into these microbes by transforming them with plasmid(s) encoding homoserine acetyltransferase, O-acetylhomoserine (thiol)-lyase, and homocysteine methylase. The parent and transformed microbes are cultivated individually in a fermentation medium containing glucose, soy hydrolysate, and inorganic nutrients. The medium is supplemented either with sulfate or sulfide as a source of sulfur for methionine production. Table I indicates the relative amount of methionine that is produced by each strain.

Table I

<u>Microbe</u>	<u>Sulfur Source</u>	<u>Methionine Produced*</u>
<u>E. coli</u> parent	sulfate	-
<u>E. coli</u> parent	sulfide	-
<u>E. coli</u> transformant	sulfate	+
<u>E. coli</u> transformant	sulfide	++
<u>C. glutamicum</u> parent	sulfate	-
<u>C. glutamicum</u> parent	sulfide	-
<u>C. glutamicum</u> transformant	sulfate	+
<u>C. glutamicum</u> transformant	sulfide	++
<u>B. flavum</u> parent	sulfate	-
<u>B. flavum</u> parent	sulfide	-
<u>B. flavum</u> transformant	sulfate	+
<u>B. flavum</u> transformant	sulfide	++

---

\* low (-), medium (+), high (++)

### Example 2

#### Homocysteine Production via Acylhomoserine (Sulphydrylation Route)

The parent strains of Example 1 are deleted for homocysteine methylase activity. The microbes are then transformed with plasmid(s) encoding homoserine acetyltransferase and O-acetylhomoserine (thiol)-lyase. The homocysteine methylase negative parent and transformed microbes are cultivated as in Example 1. Table II indicates the relative amount of homocysteine that is produced by each strain.

Table II

<u>Microbe*</u>	<u>Sulfur Source</u>	<u>Homocysteine Produced**</u>
<u>E. coli</u> parent	sulfate	-
<u>E. coli</u> parent	sulfide	-
<u>E. coli</u> transformant	sulfate	+
<u>E. coli</u> transformant	sulfide	++
<u>C. glutamicum</u> parent	sulfate	-
<u>C. glutamicum</u> parent	sulfide	-
<u>C. glutamicum</u> transformant	sulfate	+
<u>C. glutamicum</u> transformant	sulfide	++
<u>B. flavum</u> parent	sulfate	-
<u>B. flavum</u> parent	sulfide	-
<u>B. flavum</u> transformant	sulfate	+
<u>B. flavum</u> transformant	sulfide	++

\*All strains lack homocysteine methylase activity

\*\*low (-), medium (+), high (++)

### Example 3

#### Methionine Production via Acylhomoserine (Methylsulphydrylation Route)

The strains of Example 2 are cultivated as in Example 1 except that methylmercaptan is supplied as the supplemental sulfur source. Table III indicates the relative amount of methionine that is produced by each strain. Methionine production is indicative of a functioning methylsulphydrylation pathway.

Table III

<u>Microbe*</u>	<u>Methionine Produced**</u>
<u>E. coli</u> parent	-
<u>E. coli</u> transformant	++
<u>C. glutamicum</u> parent	-
<u>C. glutamicum</u> transformant	++
<u>B. flavum</u> parent	-
<u>B. flavum</u> transformant	++

\*All strains lack homocysteine methylase activity

\*\*low (-), high (++)

Example 4

Methionine Production via Phosphohomoserine  
(Sulphydrylation Route)

The parent strains of Example 1 are transformed with plasmid(s) encoding homoserine kinase, plant cystathionine  $\gamma$ -synthase and homocysteine methylase. The parent and transformed microbes are cultivated as in Example 1. Table IV indicates the relative amount of methionine that is produced by each strain.

Table IV

<u>Microbe</u>	<u>Sulfur Source</u>	<u>Methionine Produced*</u>
<u>E. coli</u> parent	sulfate	-
<u>E. coli</u> parent	sulfide	-
<u>E. coli</u> transformant	sulfate	+
<u>E. coli</u> transformant	sulfide	++
<u>C. glutamicum</u> parent	sulfate	-
<u>C. glutamicum</u> parent	sulfide	-
<u>C. glutamicum</u> transformant	sulfate	+
<u>C. glutamicum</u> transformant	sulfide	++
<u>B. flavum</u> parent	sulfate	-
<u>B. flavum</u> parent	sulfide	-
<u>B. flavum</u> transformant	sulfate	+
<u>B. flavum</u> transformant	sulfide	++

\* low (-), medium (+), high (++)

#### Example 5

##### Methionine Production via Phosphohomoserine (Methylsulfhydrylation Route)

The deleted parent strains of Example 2 are transformed with plasmid(s) encoding homoserine kinase and plant cystathionine  $\gamma$ -synthase. The parent and transformed microbes are cultivated as in Example 3. Table V indicates the relative amount of methionine that is produced by each strain.

Table V

<u>Microbe*</u>	<u>Methionine Produced**</u>
<u>E. coli</u> parent	-
<u>E. coli</u> transformant	++
<u>C. glutamicum</u> parent	-
<u>C. glutamicum</u> transformant	++
<u>B. flavum</u> parent	-
<u>B. flavum</u> transformant	++

---

\*All strains lack homocysteine methylase activity and were supplied with methylmercaptan

\*\*low (-), high (++)

#### Example 6

##### Methionine Production via Homoserine (Sulfhydrylation Route)

The parent strains of Example 1 are deleted for their homoserine acyltransferase activity. The microbes are then transformed with plasmid(s) encoding O-acetylhomoserine (thiol)-lyase from S. pombe and homocysteine methylase. The parent and transformed microbes are cultivated as in Example 1. Table VI indicates the relative amount of methionine that is produced by each strain.

Table VI

<u>Microbe*</u>	<u>Sulfur Source</u>	<u>Methionine Produced**</u>
<u>E. coli</u> parent	sulfate	-
<u>E. coli</u> parent	sulfide	-
<u>E. coli</u> transformant	sulfate	+
<u>E. coli</u> transformant	sulfide	++
<u>C. glutamicum</u> parent	sulfate	-
<u>C. glutamicum</u> parent	sulfide	-
<u>C. glutamicum</u> transformant	sulfate	+
<u>C. glutamicum</u> transformant	sulfide	++
<u>B. flavum</u> parent	sulfate	-
<u>B. flavum</u> parent	sulfide	-
<u>B. flavum</u> transformant	sulfate	+
<u>B. flavum</u> transformant	sulfide	++

---

\*All strains lack homoserine acyltransferase activity

\*\*low (-), medium (+), high (++)

Example 7

Methionine Production via Homoserine  
(Methylsulphydrylation Route)

The deleted parent strains of Example 6 are transformed with a plasmid encoding O-acetylhomoserine (thiol)-lyase from S. pombe. The parent and transformed microbes are cultivated as in Example 3. Table VII indicates the relative amount of methionine that is produced by each strain.

Table VII

<u>Microbe*</u>	<u>Methionine Produced**</u>
<u>E. coli</u> parent	-
<u>E. coli</u> transformant	++
<u>C. glutamicum</u> parent	-
<u>C. glutamicum</u> transformant	++
<u>B. flavum</u> parent	-
<u>B. flavum</u> transformant	++

---

\*All strains lack homoserine acyltransferase activity

\*\*low (-), high (++)

WHAT IS CLAIMED IS:

1. A method for enhancing methionine production in a fermentation process of a microbial cell by modifying the methionine biosynthetic pathway of said cell comprising the steps of:
  - i. transforming or transducing a homoserine-activating enzyme gene fragment capable of expressing said homoserine-activating enzyme and a sulfur-incorporating enzyme gene fragment capable of expressing said sulfur enzyme into said cell;
  - ii. growing said cell under such conditions that transformation or transduction of both gene enzymes are effected;
  - iii. recovering a transformed or transduced cell; and
  - iv. adding an exogenous sulfur compound other than cysteine or methionine to said transformed or transduced cell as the sulfur source for methionine production.
2. The method of claim 26 wherein said exogenous sulfur compound is a reduced sulfur compound consisting of hydrogen sulfide, methyl mercaptan or a salt thereof.
3. The method of claim 26 wherein said exogenous sulfur compound is an oxidized sulfur compound consisting of sulfate, sulfite or thiosulfate.
4. The method of claim 27 or 28 wherein said homoserine-activating enzyme is selected from the group consisting of homoserine kinase, homoserine acetyltransferase and homoserine succinyltransferase.
5. The method of claim 27 or 28 wherein said sulfur-incorporating enzyme is selected from the group consisting of O-succinylhomoserine (thiol)-lyase, O-acetylhomoserine (thiol)-lyase and plant cystathionine gamma synthase.
6. The method of claim 27 wherein said sulfur-incorporating enzyme converts homoserine and said hydrogen sulfide or a salt thereof directly to homocysteine.
7. The method of claim 27 wherein said sulfur-incorporating enzyme converts homoserine and said methyl mercaptan or a salt thereof directly to methionine.

8. The method of claim 28 wherein said sulfur-incorporating enzyme converts homoserine directly to homocysteine.
9. A method for enhancing homocysteine production in a fermentation process of a microbial cell by modifying the methionine biosynthetic pathway of said cell comprising the steps of:
- i. transforming or transducing a homoserine-activating enzyme gene fragment capable of expressing any said homoserine-activating enzyme but not including homocysteine methylase, and a sulfur-incorporating enzyme gene fragment capable of expressing said sulfur enzyme into said cell;
  - ii. growing said cell under such conditions that transformation or transduction of both gene enzyme fragments are effected;
  - iii. recovering a transformed or transduced cell; and
  - iv. adding an exogenous sulfur compound other than methionine or cysteine to the said transformed or transduced cell as the sulfur source for homocysteine production.
10. The method of claim 34 wherein said exogenous sulfur compound is a reduced sulfur compound consisting of hydrogen sulfide, or a salt thereof.
11. The method of claim 34 wherein said exogenous sulfur compound is an oxidized sulfur compound consisting of sulfate, sulfite or thiosulfate.
12. The method of claim 35 or 36 wherein said homoserine-activating enzyme is selected from the group consisting of homoserine kinase, homoserine acetyltransferase and homoserine succinyltransferase.
13. The method of claim 35 or 36 wherein said sulfur-incorporating enzyme is selected from the group consisting of O-succinylhomoserine (thiol)-lyase, O-acetylhomoserine (thiol)-lyase and plant cystathionine gamma synthase.
14. The method of claim 35 wherein said sulfur-incorporating enzyme converts homoserine and said hydrogen sulfide or a salt thereof directly to homocysteine.
15. The method of claim 36 wherein said sulfur-incorporating enzyme converts homoserine directly to homocysteine.

16. The method of claim 26 or 34 wherein said transformed or transduced cell produces an amino acid that is greater than said amino acid of a non-transformed or transduced cell.

17. The method of claim 26 or 34 wherein said transformed or transduced cell is selected from the group consisting of *Corynebacteria*, *Brevibacteria* or *Escherichia coli*.



# AMENDED CLAIMS

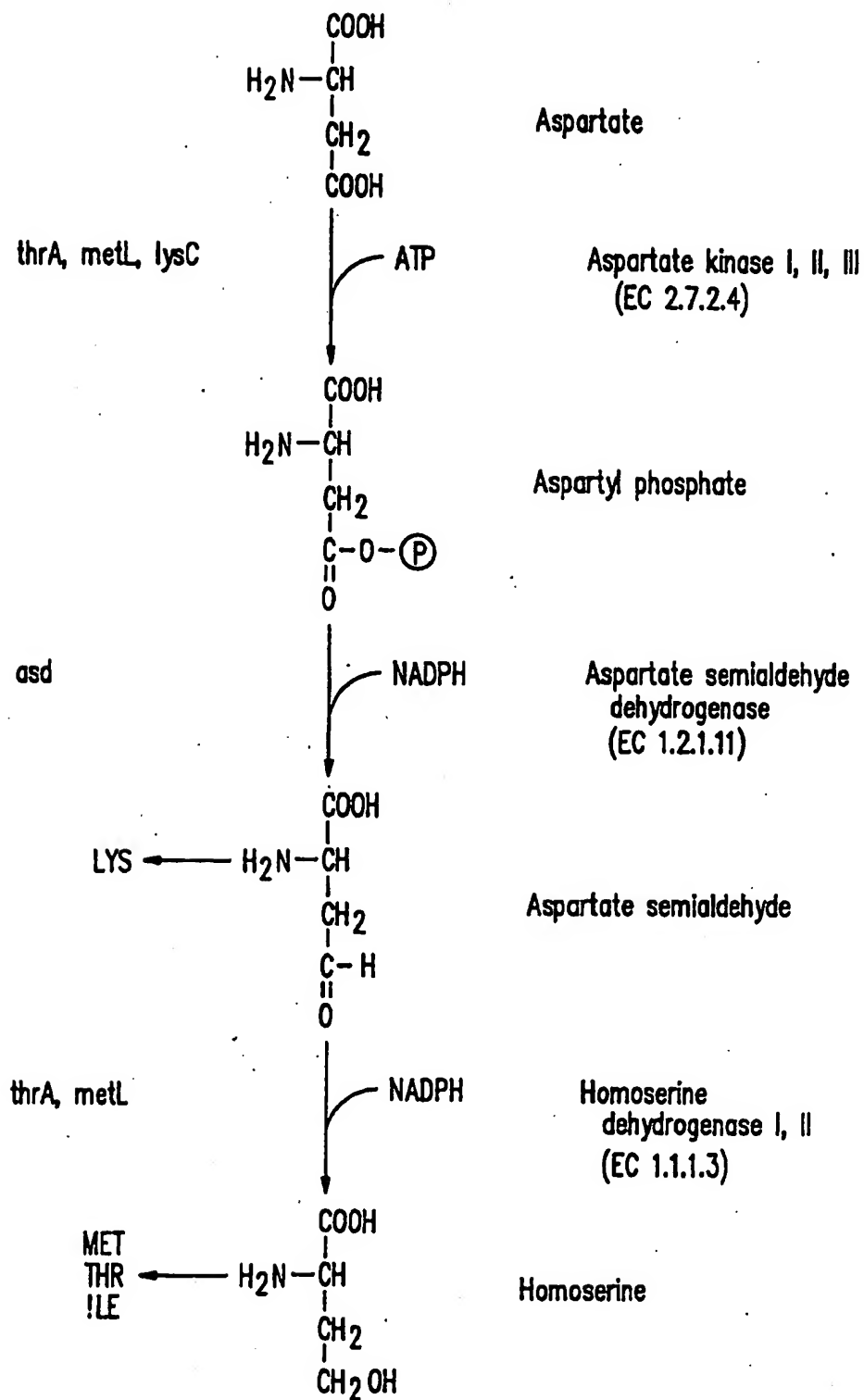
[received by the International Bureau on 3 August 1993 (03.08.93);  
original claims 2-8, 10 and 17 amended; other claims unchanged (3 pages)]

1. A method for enhancing methionine production in a fermentation process of a microbial cell by modifying the methionine biosynthetic pathway of said cell comprising the steps of:
  - i. transforming or transducing a homoserine-activating enzyme gene fragment capable of expressing said homoserine-activating enzyme and a sulfur-incorporating enzyme gene fragment capable of expressing said sulfur enzyme into said cell;
  - ii. growing said cell under such conditions that transformation or transduction of both gene enzymes are effected;
  - iii. recovering a transformed or transduced cell; and
  - iv. adding an exogenous sulfur compound other than cysteine or methionine to said transformed or transduced cell as the sulfur source for methionine production.
2. The method of claim 1 wherein said exogenous sulfur compound is a reduced sulfur compound consisting of hydrogen sulfide, methyl mercaptan or a salt thereof.
3. The method of claim 1 wherein said exogenous sulfur compound is an oxidized sulfur compound consisting of sulfate, sulfite or thiosulfate.
4. The method of claim 2 or 3 wherein said homoserine-activating enzyme is selected from the group consisting of homoserine kinase, homoserine acetyltransferase and homoserine succinyltransferase.
5. The method of claim 2 or 3 wherein said sulfur-incorporating enzyme is selected from the group consisting of O-succinylhomoserine (thiol)-lyase, O-acetylhomoserine (thiol)-lyase and plant cystathionine gamma synthase.
6. The method of claim 2 wherein said sulfur-incorporating enzyme converts homoserine and said hydrogen sulfide or a salt thereof directly to homocysteine.
7. The method of claim 2 wherein said sulfur-incorporating enzyme converts homoserine and said methyl mercaptan or a salt thereof directly to methionine.

8. The method of claim 3 wherein said sulfur-incorporating enzyme converts homoserine directly to homocysteine.
9. A method for enhancing homocysteine production in a fermentation process of a microbial cell by modifying the methionine biosynthetic pathway of said cell comprising the steps of:
- i. transforming or transducing a homoserine-activating enzyme gene fragment capable of expressing any said homoserine-activating enzyme but not including homocysteine methylase, and a sulfur-incorporating enzyme gene fragment capable of expressing said sulfur enzyme into said cell;
  - ii. growing said cell under such conditions that transformation or transduction of both gene enzyme fragments are effected;
  - iii. recovering a transformed or transduced cell; and
  - iv. adding an exogenous sulfur compound other than methionine or cysteine to the said transformed or transduced cell as the sulfur source for homocysteine production.
10. The method of claim 9 wherein said exogenous sulfur compound is a reduced sulfur compound consisting of hydrogen sulfide, or a salt thereof.
11. The method of claim 9 wherein said exogenous sulfur compound is an oxidized sulfur compound consisting of sulfate, sulfite or thiosulfate.
12. The method of claim 10 or 11 wherein said homoserine-activating enzyme is selected from the group consisting of homoserine kinase, homoserine acetyltransferase and homoserine succinyltransferase.
13. The method of claim 10 or 11 wherein said sulfur-incorporating enzyme is selected from the group consisting of O-succinylhomoserine (thiol)-lyase, O-acetylhomoserine (thiol)-lyase and plant cystathionine gamma synthase.
14. The method of claim 10 wherein said sulfur-incorporating enzyme converts homoserine and said hydrogen sulfide or a salt thereof directly to homocysteine.
15. The method of claim 11 wherein said sulfur-incorporating enzyme converts homoserine directly to homocysteine.

16. The method of claim 1 or 9 wherein said transformed or transduced cell produces an amino acid that is greater than said amino acid of a non-transformed or transduced cell.

17. The method of claim 1 or 9 wherein said transformed or transduced cell is selected from the group consisting of *Corynebacteria*, *Brevibacteria* or *Escherichia coli*.



**FIG. 1a**

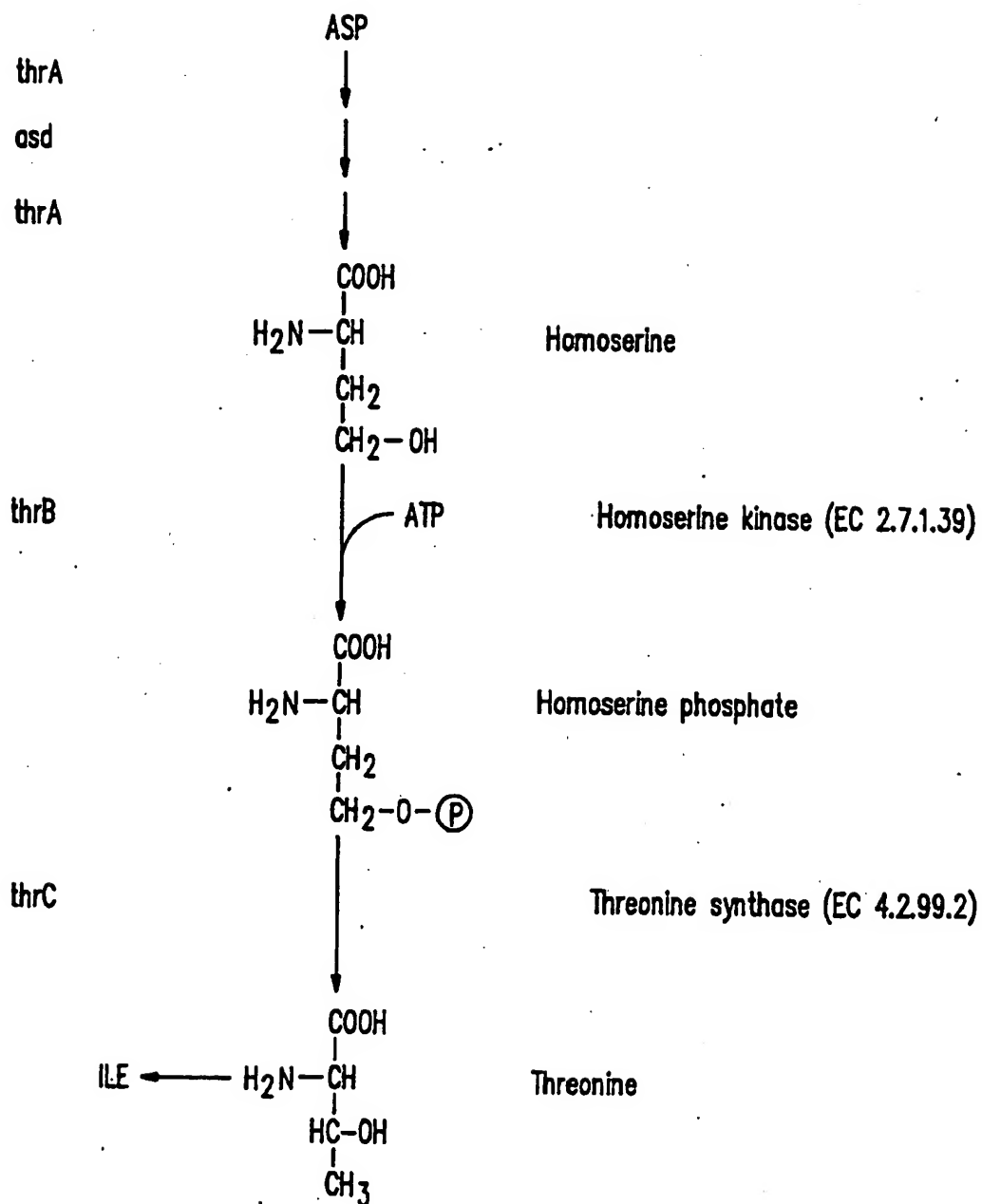
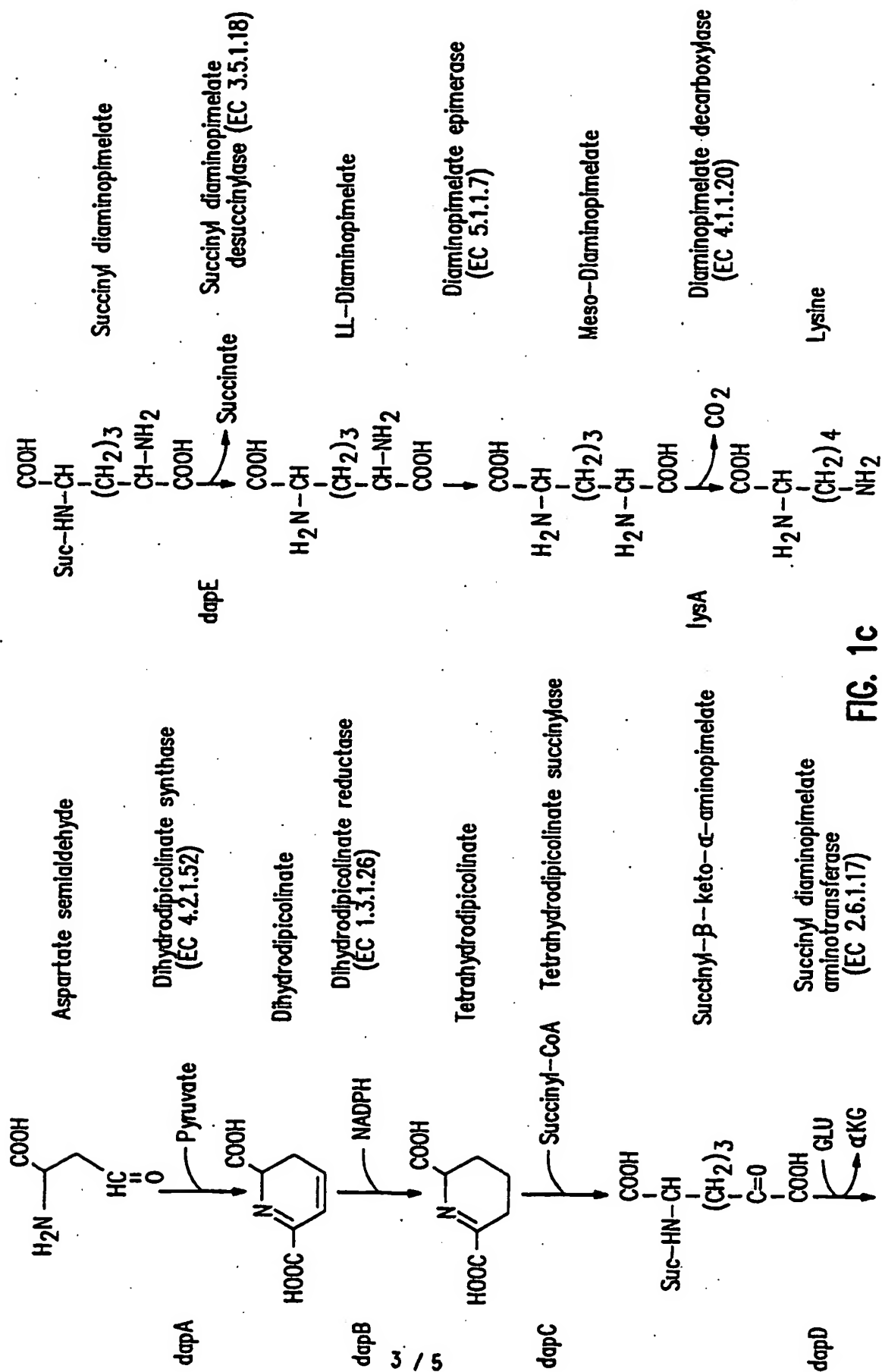
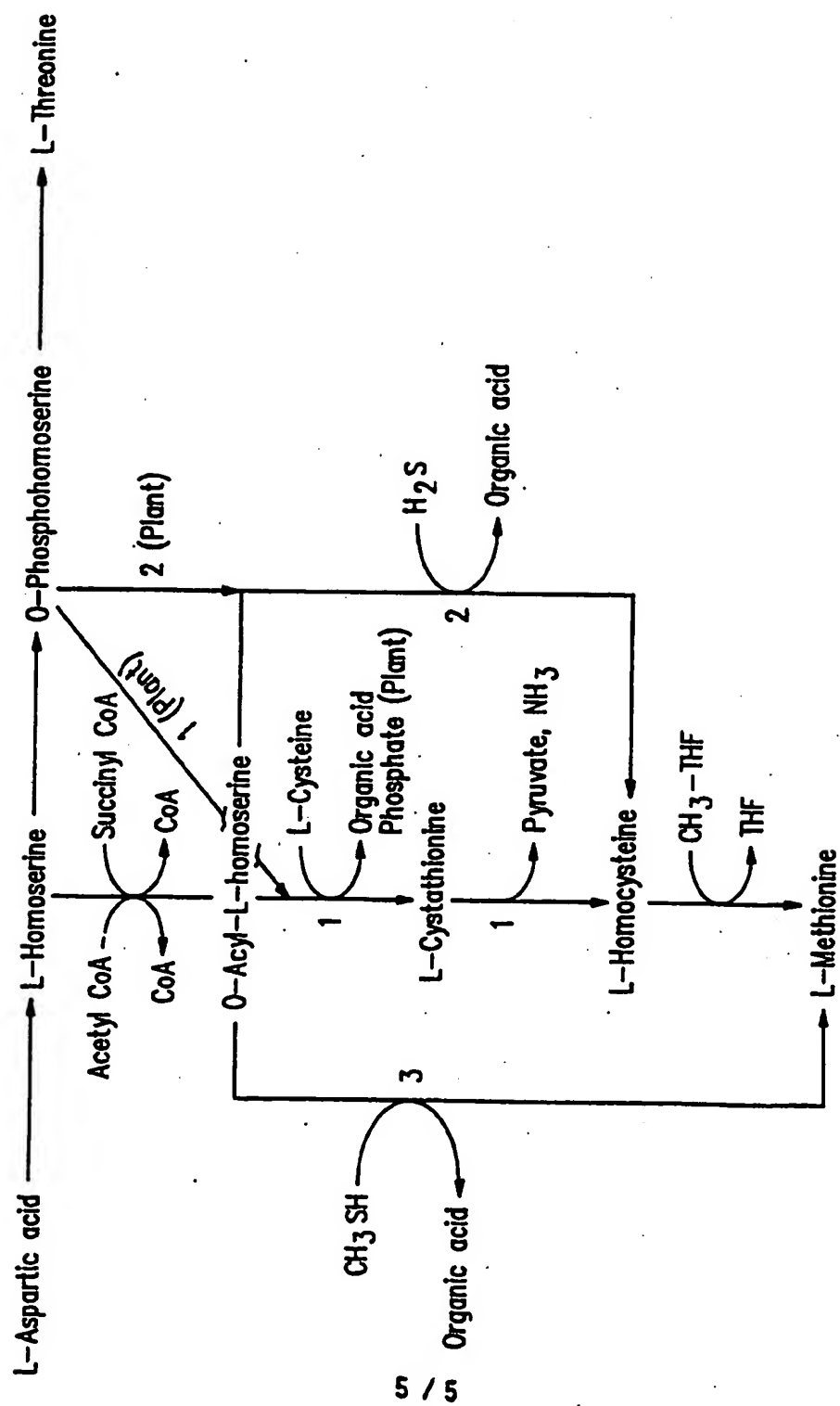


FIG. 1b  
2 / 5



**FIG. 1c**





**FIG. 2**



<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/54;                      C12P13/12;                      C12N15/60;                      C12P13/04		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12P ;                      C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	<p>CHEMICAL ABSTRACTS, vol. 98, no. 11, 14 March 1983, Columbus, Ohio, US; abstract no. 85955m, SIMON, MARCIA ET AL 'Direct homocysteine biosynthesis from O-succinylhomoserine in Escherichia coli : An alternate pathway that bypasses cystathionine.' page 275 ;column R ; see abstract &amp; JOURNAL OF BACTERIOLOGY vol. 153, no. 1, 1983, pages 558 - 561</p> <p style="text-align: center;">-----</p>	
<p><sup>10</sup> Special categories of cited documents :<sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"d" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
15 JUNE 1993		02 -07- 1993
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		LE CORNEC N.D.R.

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
**OBSCURITIES.**  
See additional page
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

OBSCURITIES

There is a mistake in the wording of the dependant claims.  
The claims have been understood and searched as follows:

- a) claim 1 has been assumed to be equivalent to claim "26",  
and dependant claims 2-8 have therefore been assumed  
to be equivalent to claims "27-33";
- b) claim 9 has been assumed to be equivalent to claim "34",  
and dependant claims 10-17 have therefore been assumed  
to be equivalent to claims "35-42".